28 December 2004

Re: Einat *et al.*; Application No. 09/802,472
"Sequences characteristic of hypoxia regulated gene transcription"

DECLARATION

1. My name is Dr. Peter Chumakov, and I have worked closely with the inventors of the above-referenced patent application on experimentation relating to said invention.

2. In the above-referenced application, the following statements were made by the inventors:

   a) On page 38 line 21 to page 39 line 6, it was stated that "Bad genes are useful in that they can be used in diagnostic assays for cells that have been subjected to hypoxia or ischemia. If mRNA corresponding to such genes, or the translation product thereof, is found in the cells being assayed it is likely that they have been subjected to hypoxia or ischemia. If diagnosed pre-stroke, this may be predictive of incipient stroke. They are also useful as a target for assays for the discovery of drugs which selectively down-regulate such genes or are otherwise dominant negative with respect to the expression of the gene product of such genes."

   b) On page 40 line 17 to page 41 line 8, it was stated that "As all of the genes of the present invention have been found to be modulated significantly upward after the cells have been subject to hypoxia, all of such genes may be

considered to be a gene of interest for the purpose of the diagnostic assays reported herein. Methods of detecting tissue hypoxia in mammalian tissue are based on the use of the mRNA of the genes of interest or the translation product thereof as a diagnostic marker for cells that have been subjected to hypoxia or ischemia. It is possible to determine the level of the mRNAs or protein translation products corresponding to these bad genes, in normal tissue or bodily fluids as compared to hypoxic tissue a bodily fluid from a subject which has suffered a hypoxic event, and, thus, determine the reference values of these genes on mRNAs or proteins which are indicative of tissue hypoxia".

3. Under my direction and control, a series of experiments has been performed as follows:

(a) Polyclonal antibodies against gene 95 (also referred to herein as "Hi95" "for Hypoxia induced (gene) 95"), polypeptide were generated and tested for specificity (details are presented in the Appendix, Experiment A).

(b) An experiment was designed and conducted in order to verify the induction of the expression of the gene 95 polypeptide as a result of oxidative stress events (details are presented in the Appendix, Experiment B).

(c) An experiment was designed and conducted in order to verify the induction of the expression of the gene 95 polypeptide as a result of DNA damage (details are presented in the Appendix, Experiment C).

4. From the above experiments, the following conclusions can be drawn:

a) Affinity purified antibodies against the gene 95 polypeptide recognize both the exogenous (Figure 1) and the endogenous gene 95 human polypeptide (Figures 2 and 3) with high specificity.

b) Elevated expression of the gene 95 polypeptide following treatment of cells with H_2O_2 was detected in all cell lines tested (Figure 2).

c) Induction of gene 95 polypeptide expression was completely abolished in RKO cells, in which p53 expression was inactivated via RNA interference. In contrast, both types of cells retained the ability to strongly induce the gene 95 polypeptide in response to H_2O_2 (Figure 3).

d) Thus, in addition to elevation of gene 95 mRNA levels, the expression of the gene 95 polypeptide is indeed induced in Humans in response to oxidative stress.

5. Thus, these experiments provide further experimental evidence for the statements described in item 2 above, i.e., that the gene 95 polypeptide is useful for diagnostic assays.

APPENDIX

EXPERIMENT A

Generation of rabbit polyclonal antibodies against Hi95 (gene 95) polypeptide

Polyclonal anti human-Hi95 antibodies were generated by immunization of rabbits with a bacterially expressed fragment of the human Hi95 polypeptide encompassing amino acids 100-435. The polypeptide fragment contained an N-terminal histidine tag (His_{10}-$Hi95_{100-435}$ fusion protein). Prior to injection into rabbits, the antigen was affinity purified using Ni-columns. Testing of the antibodies generated can be seen in Figure 1.

EXPERIMENT B

Expression of the human Hi95 polypeptide is induced by oxidative stress in various human cell lines

A number of different human cancer cell lines were treated with 0.5 M H_2O_2 for 24 hours in vitro or left untreated for the same period of time. Total protein extracts were separated on 10% polyacrylamide gels, transferred to nitrocellulose membrane and immunoblotted with anti-Hi95 antibodies. The results of these experiments can be seen in Figure 2.

EXPERIMENT C

Expression of the Hi95 polypeptide is induced in response to DNA damage in a p53-dependent manner.

P53-positive RKO cells were infected with a lentiviral vector expressing shRNA against p53 or with an empty vector as a control. Isogenic cell pairs were subjected to doxorubicin (50 ng/ml) or UV treatment, which confer genotoxic stress. Induction of Hi95 polypeptide expression was completely abolished in the RKO cells, in which p53 expression was inactivated via RNA interference. In contrast, both types of cells retained the ability to strongly induce Hi95 protein in response to H_2O_2. The results of these experiments are demonstrated in Figure 3.

FIGURES

Figure 1

Immunoblotting of protein lysates of Hi95 infected Rat-1cells

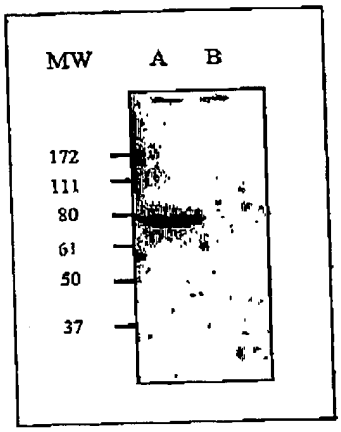

Figure 2

Immunoblotting of protein extracts derived from various cell lines before and after oxidative stress treatment with anti-Hi95 antibodies

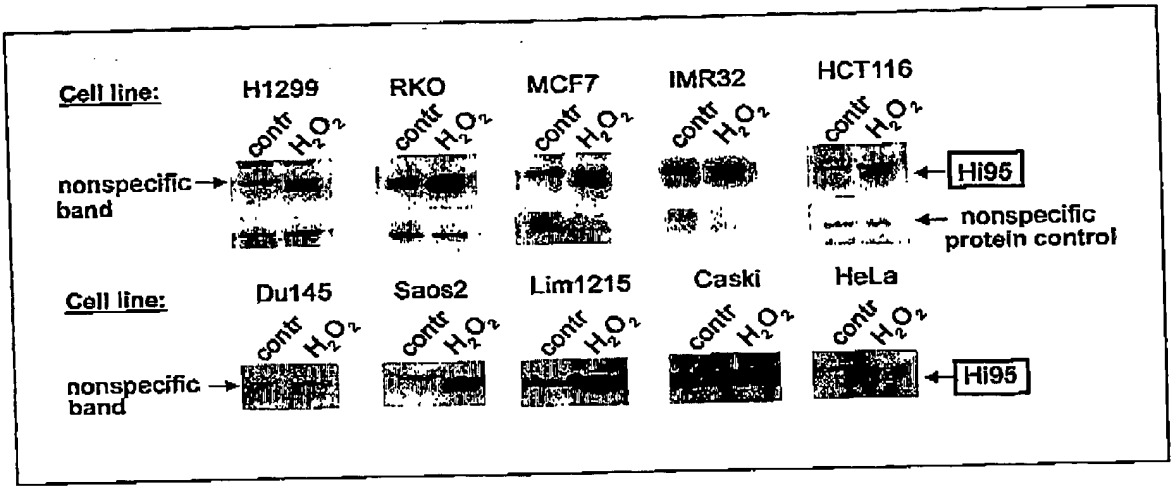

Figure 3

Immunoblotting of protein extracts derived from isogenic p53-positive and p53-negative RKO cells before and after genotoxic stress treatments with anti-Hi95 antibodies

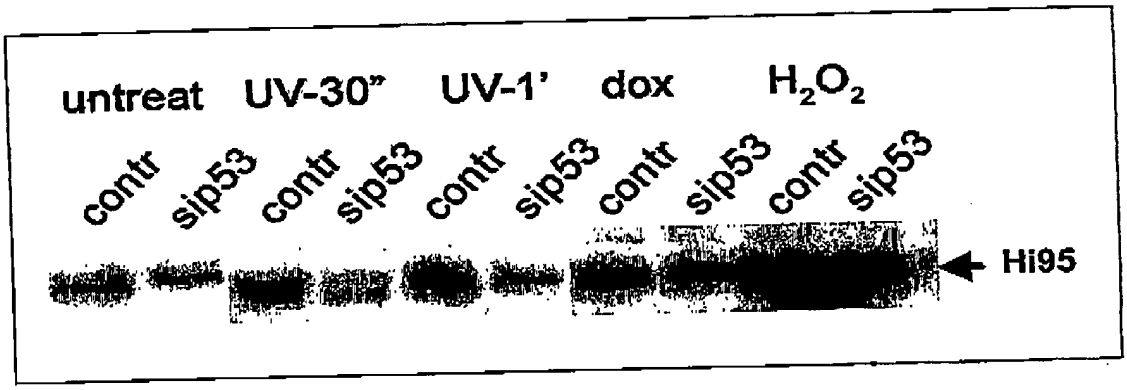

FIGURE LEGENDS

Figure 1

Immunoblotting of protein lysates of Hi95 infected Rat-1cells 24h after infection (Lane A-10μg/lane) and pBabe (empty vector) infected Rat-1 cells (Lane B - 10μg/lane), with αHP95 (lot #AP30902301) 1μg/ml. MW markers: kDa.

Figure 3

Immunoblotting with anti-Hi95 antibodies of protein extracts from isogenic p53-positive and p53-negative RKO cells. Contr – RKO cells infected with empty lentiviral vector. sip53- RKO cells infected with lentivirus expressing shRNA against p53. The treatments are indicated over the blot.